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Ethanol metabolism in a peroxisome-deficient mutant of the yeast *Hansenula polymorpha*

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1. SUMMARY

This paper describes ethanol metabolism in a peroxisome-deficient (PER) mutant of *Hansenula polymorpha*. The PER mutant was able to use ethanol as sole-carbon source but showed reduced growth rates compared to wild-type cells together with a reduced rate of ethanol utilization under μ_{max} conditions. In chemostat cultures at low-dilution rates, the activities of alcohol dehydrogenase, isocitrate lyase and malate synthase were comparable in wild-type and PER cells. In PER cells the two latter enzymes, exclusively microbody-bound in wild-type cells, were active in the cytosol. The possible advantage of intact microbodies in the intermediary metabolism of ethanol in *H. polymorpha* is discussed.

2. INTRODUCTION

During growth of yeasts on various organic carbon and/or nitrogen sources, microbodies (peroxisomes, glyoxysomes) play an indispensible role in that they contain key enzymes involved in the metabolism of these compounds [1–3]. Recently, different peroxisome-deficient (PER) mutants of the yeasts *Saccharomyces cerevisiae* and *Hansenula polymorpha* have been isolated [4,5]. Screening of these mutants was based on their inability to grow on certain carbon sources, the metabolism of which is mediated by peroxisomal matrix enzymes. However, further studies indicated that the absence of intact peroxisomes did not result in a general impairment of peroxisomal metabolic functions. For example, peroxisome-deficient mutants of *H. polymorpha* grow at rates comparable to those of wild-type (WT) cells on different organic nitrogen sources, such as primary amines and D-amino acids [6].

Preliminary experiments indicated that PER mutants could also utilize ethanol as the sole-carbon and energy source. This was unexpected since in WT cells microbodies (glyoxysomes) contain the key enzymes of the glyoxylate cycle, isocitrate lyase (ICL) and malate synthase (MS) and so play an essential role in intermediary ethanol metabolism. For this reason we have studied the metabolism of this C2-compound in a

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PER mutant of *H. polymorpha* in more detail. The results of this study are given below.

3. MATERIALS AND METHODS

3.1. Microorganisms and growth conditions

Both WT *Hansenula polymorpha* (CBS 4732) and a PER-mutant derived from this strain (strain 125-2E [5]), were grown in batch cultures at 37 °C in minimal medium [7], supplemented with 0.5% (w/v) glucose or 0.5% (v/v) ethanol as sole-carbon source. Both WT and PER mutant were also grown in ethanol-limited (0.5% (v/v)) continuous cultures [8], both connected to the same medium reservoir. Growth is expressed as absorbance of the culture measured at 660 nm (A_{660}).

3.2. Enzyme assays

Cell free extracts were prepared as described [7]. Enzyme assays were performed by published methods: cytochrome C oxidase [9], catalase [10], isocitrate lyase (ICL) [11], malate synthase (MS) [12], malate dehydrogenase (MDH) [13], glutamate oxaloacetate aminotransferase (GOT) [14], glutamate dehydrogenase (GDH) [14], alcohol dehydrogenase (ADH) [15]. Enzymic activities are expressed as μmol substrate consumed or product formed min^{-1} mg^{-1} protein; catalase as ΔE_{240} min^{-1} mg^{-1} protein. Protein [16] and ethanol [17] concentrations were measured as described previously. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and Western blotting were carried out as already described [6]. The native size of MS was determined by sucrose-velocity centrifugation and FPLC, using a Superose 6 gel-filtration column [18]. For cell-fractionation studies, protoplasts were homogenized and subjected to differential centrifugation [9]. The $30\,000 \times g$ pellet (P4) and supernatant (S4) were used for enzyme analysis. Immunocytochemical experiments were performed on ultrathin sections with specific antibodies against MS [6]. Glycogen was stained on ultrathin sections [19].

4. RESULTS

Growth and enzyme patterns of different enzymes involved in ethanol metabolism of WT and PER mutant of *H. polymorpha* are summarized in Table 1. In batch cultures, growth of the PER mutant on ethanol, but not on glucose, was reduced compared with WT cells. In crude extracts of cells from the mid-exponential-growth phase on ethanol of both strains, the activities of ICL and MS were comparable (Table 1).

A significant difference between WT and PER cultures was observed with respect to the rate of ethanol consumption under μ_{max} conditions (Fig. 1). In batch cultures, cells of the PER-mutant utilized ethanol with lower efficiency, reflected in a reduced yield, compared with WT cells (Fig. 1). Also, PER cells contained considerable amounts of glycogen (Fig. 2), which was absent in WT cells. In order to further clarify the apparent difference in specific-ethanol-utilization rate in WT and PER cells, both strains were grown in ethanol-limited-continuous cultures at different dilution rates (D). The results for two extremes, namely at low-D (0.04 h^{-1}) and high-D (0.14 h^{-1}), are summarized in Table 2, showing that

Table 1

Growth and activities of ADH and different microbody matrix enzymes in cells of WT and the PER mutant of *H. polymorpha*, grown in batch cultures on ethanol or glucose as carbon (C) source

Strain	C-source	cat	ICL	MDH	MS	ADH	t_d (h)
WT	Glucose	15.1	0.03	21.3	0.36	15.0	1.2
WT	Ethanol	17.4	0.19	39.1	1.31	15.4	2.2
PER	Glucose	27.9	0.02	35.9	0.34	16.0	1.3
PER	Ethanol	40.5	0.17	80.4	1.24	8.9	3.2

Cells were harvested from the mid-exponential growth phase. Growth is expressed as doubling time (h).

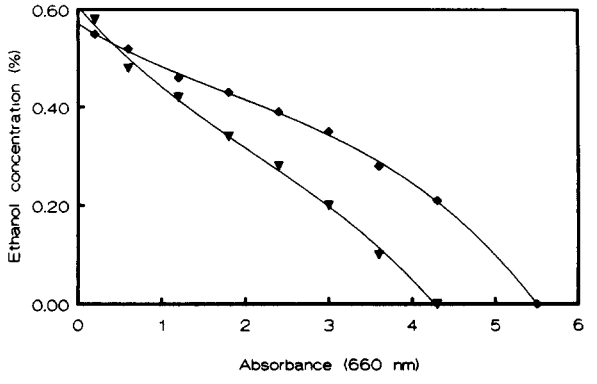

Fig. 1. Ethanol consumption as function of the cell mass (expressed as A_{660}) in batch cultures of WT (♦) and PER mutant (▼) of *H. polymorpha*.

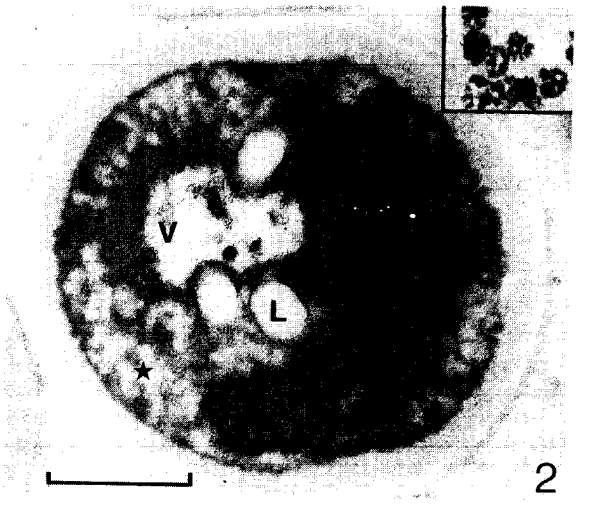

Fig. 2. Thin section of a PER-cell from a batch culture in the late-exponential growth phase on ethanol, showing the accumulation of glycogen (*). Inset: staining of glycogen granules. N, nucleus; V, vacuole; L, lipid droplet; bar = 0.5 μm.

for WT cells an increase in D is associated with a minor increase in cell yield. The density of the PER culture, originally comparable to WT levels at low D, gradually decreased with increasing D (Table 2). At Ds ranging from 0.04–0.14 h^{-1} cultures from WT cells invariably were carbon-limited; ethanol was not detectable in the culture fluid. Similar results were obtained with the PER strain at low D (0.04 h^{-1}). The decrease in density of the PER cultures at higher D was associated with the appearance of increasing amounts of ethanol in the culture fluid. At $D = 0.14$ h^{-1}, approximately 26% of the ethanol substrate, originally present in the feed, was not used and was present in the culture vessel. The activities of ADH and different microbody enzymes were determined in crude extracts prepared from cells grown at $D = 0.04$ or 0.14 h^{-1}, respectively (Table 2). At $D = 0.04$ h^{-1} the levels of these enzymes were comparable in WT and PER mutant. However, at $D = 0.14$ h^{-1}, the overall activities were lower in the PER strain, compared with $D = 0.04$ h^{-1}, except for MDH and GOT. The decrease in specific activity of ADH in the PER mutant was not caused by an inactivation of the enzyme but, rather, by a reduction in the amount of enzyme protein, as was evident after Western blotting (Fig. 3). Similar results were obtained for MS (not shown). Also, the molecular mass of native MS protein was identical in WT and PER mutant and amounted approximately to 120 kD, as was indicated after FPLC gel filtration (Fig. 4) and Western blotting (Fig. 4, inset). The native molecular mass of 120 kD was confirmed by sucrose-velocity centrifugation, which indicated that MS assembled into a dimer in both strains. These results are in contrast to the data reported

Table 2

Cell yield and activities of ADH and different microbody matrix enzymes in cells of WT and PER-mutant of *H. polymorpha*, grown on ethanol in continuous cultures at different dilution rates (D)

Strain	D (h^{-1})	cat	ICL	MDH	MS	ADH	GOT	GDH	Yield
WT	0.04	23.3	0.05	49.2	0.81	26.3	0.11	1.01	7.2
PER	0.04	48.3	0.09	18.2	0.84	25.0	0.09	0.89	6.8
WT	0.14	23.7	0.13	45.8	1.72	69.2	0.08	1.12	7.8
PER	0.14	18.5	0.05	84.7	0.36	4.3	0.16	0.55	2.9

Cell yield is expressed as A_{660}.

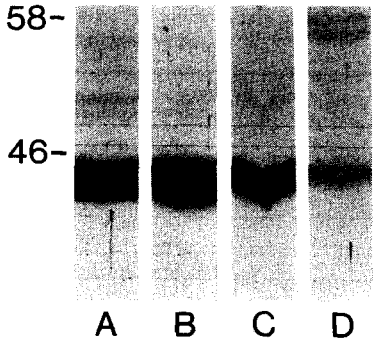

Fig. 3. Western blots, using specific antibodies against ADH, of crude extracts prepared from ethanol-grown cells at low-dilution rate ($D = 0.04\ h^{-1}$, lanes **A**, **B**) and high-dilution rate ($D = 0.14\ h^{-1}$, lanes **C**, **D**) from WT (lanes **A**, **C**) and PER mutant (lanes **B**, **D**). Molecular mass indicated in kD.

by Bruinenberg et al. [20], which suggested that MS assembled into a tetramer in WT *H. polymorpha*. Different enzymes which are microbody-

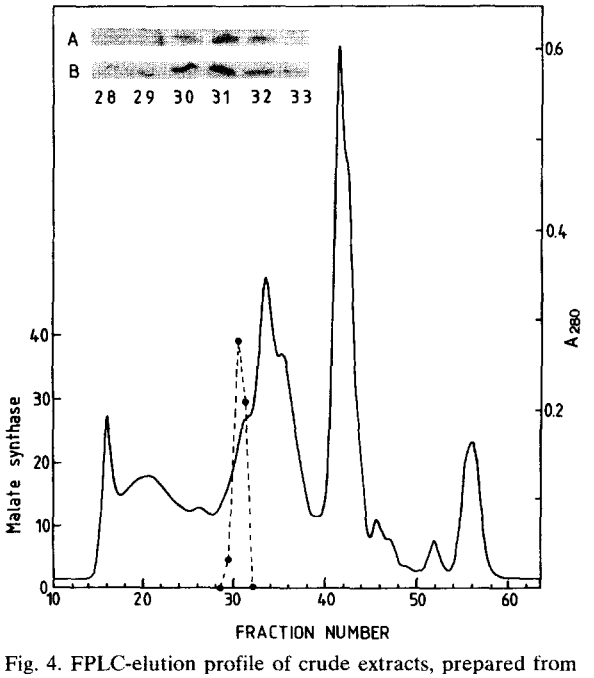

Fig. 4. FPLC-elution profile of crude extracts, prepared from ethanol-grown PER cells. MS activity (dotted line) was only detected in fractions 29–32. All fractions obtained (0.5 ml) were subjected to Western blotting; characteristic examples are shown as inset **A** at the top. Inset **B** represents a Western blot of identical fractions obtained from WT *H. polymorpha*. MS activity is expressed at U ml^{-1}.

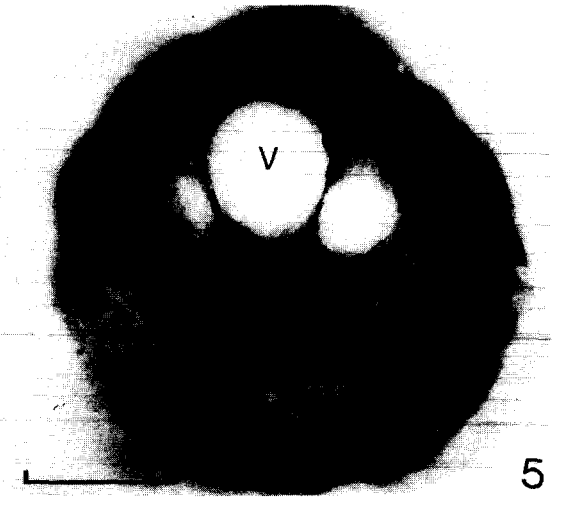

Fig. 5. Immunocytochemical demonstration of MS protein in the cytosol of an ethanol-grown cell of the PER-mutant of *H. polymorpha* (continuous culture at $D = 0.04\ h^{-1}$, α MS-protein A/ gold). (M, mitochondrion; V, vacuole; bar = 0.5 μm).

bound in WT cells of *H. polymorpha* are located in the cytosol of PER cells [5]. This location was confirmed in chemostat-grown cells, used in the present study by immunocytochemical (Fig. 5) and conventional fractionation methods (not shown). ADH is also located in the cytosol of both strains, as was indicated by the ratio of the specific activities of the enzyme, detected in the S4 and P4 fractions after differential centrifugation (S4/P4 ratio = 5.8). As expected, some MDH was sedimentable (S4/P4 ratio = 3.2), because it is partly located in mitochondria [21].

5. DISCUSSION

As suggested previously [5], the absence of intact microbodies does not abolish growth of a PER mutant of *H. polymorpha* on ethanol as the sole-carbon source. The present results on ethanol utilization by this mutant adds to a further understanding of the specific significance of microbodies as functional metabolic compartments. Previous studies on peroxisome-deficient yeast strains of *H. polymorpha* and *S. cerevisiae* [4–6,22] indicated that all microbody matrix enzymes studied so far, including the enzymes indicated in this

study, may also be active in the cytosol. There is as yet no explanation of why yeast cells maintain microbodies as functional entities. As discussed previously [6,22], we have no indications that intact organelles are essential for enzyme maturation or activation. Therefore, the advantage for the cell to compartmentalize, at least partly, certain metabolic pathways in microbodies, may be related to the fact that this localization enables a more careful control of the flow of different metabolic fluxes. This assumption is not only hypothetical, as became clear in earlier studies which revealed that intact microbodies (peroxisomes) are indispensible for growth of yeasts on methanol [9] or oleic acid [4] as sole-carbon source. The impairment of growth of PER mutants of *H. polymorpha* on methanol is shown to be related to the energetic disadvantages of an altered H_2O_2-metabolism; this alteration is caused by the absence of peroxisomes, which permits the cell to appropriately control the fluxes of formaldehyde, generated from methanol, over dissimilatory and assimilatory pathways [22]. On the other hand, PER mutants grow on glucose in the presence of organic nitrogen sources, the metabolism of which is mediated by peroxisome-born oxidases [6]. In these conditions the disadvantages of the altered H_2O_2-metabolism are thought to be of minor significance since utilization of these substrates remains limited to levels just sufficient to satisfy the cellular nitrogen requirements [6].

Our present results on ethanol utilization indicate the reasons why the PER mutant is not able to maintain a high cell density on this compound in continuous cultures at higher growth rates ($D > 0.1\ h^{-1}$). There must also be reasons related to the inefficiency of a particular metabolic pathway in the absence of microbodies, rather than to the effect of an injurious compound. The current view on the metabolic significance of these organelles in C2-metabolism of WT yeast cells is depicted in Fig. 6. This model, originally proposed for ethylamine-grown *Trichosporon cutaneum* [23], is probably also valid for *H. polymorpha*, since all enzymes participating in this pathway have been shown to be located also in *Hansenula* microbodies [1]. The observed reduc-

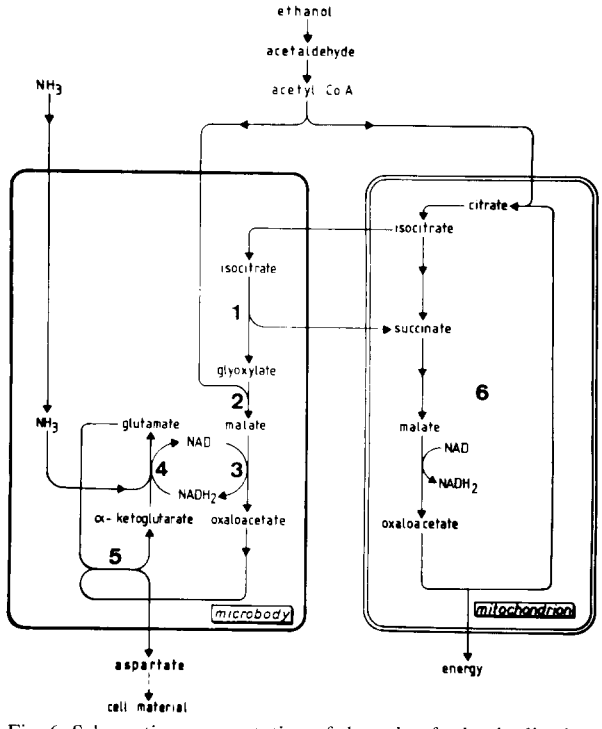

Fig. 6. Schematic representation of the role of microbodies in ethanol-metabolism in *H. polymorpha*. 1, ICL; 2, MS; 3, MDH; 4, GDH; 5, GOT; 6, TCA cycle.

tion in cell density during growth of the PER mutant on ethanol at μ_{max} conditions, parallelled by the accumulation of polyglucose, suggests that, in the absence of microbodies, the amino acid (aspartate) biosynthesis is especially hampered, most probably because the focal concentrations of the different components of the GOT enzyme reaction cannot be maintained at optimal levels. Oxaloacetate, in particular, a major intermediate in different metabolic pathways, is easily drained into other metabolic pathways (e.g. that of gluconeogenesis), thus explaining the accumulation of glycogen in the PER mutant. This hypothesis may also explain the enhanced levels of MDH observed in the mutant cells as a response of the cell to compensate oxaloacetate depletion for biosynthetic purposes.

In conclusion, we suggest that the main advantage of intact microbodies in C2-metabolism of *H. polymorpha* includes enabling the cell to adjust precisely the levels of different intermediates re-

quired for specific metabolic pathways. Another possible advantage, compartmentalizing of H_2O_2 metabolism (e.g. for utilizing the C2-nitrogen source ethylamine), is less certain in view of recent experimental data [6,24].

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